IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: WALLACH1D

In re Application of:	)	Art Unit: 1646
	)	
David WALLACH, et al.	)	Examiner: J. Dong
	)	
Appln. No.: 10/036,434	)	Washington, D.C.
	)	
Date Filed: January 7, 2002	)	Confirmation No.: 4966
	)	
For: TUMOR NECROSIS FACTOR INHIBITORY PROTEIN …	)	

SECOND DECLARATION UNDER 37 CFR §1.132 OF RIK DERYNCK

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
Customer Service Window
Randolph Building, Mail Stop
401 Dulany Street
Alexandria, VA 22314

Sir:

I, the undersigned Rik Derynck, Ph.D., hereby declare and state as follows.

I am the same Rik Derynck who executed a declaration on February 21, 2007, that was filed in this case on February 23, 2007. My qualifications and Curriculum Vitae are detailed in the original declaration.

I have been informed that the examiner has criticized my declaration, stating that "the declaration is largely opinion only, and presents no further factual

support." I further understand that the examiner stated, in the Advisory Action of May 20, 2007:

> Dr. Derynck does not base his opinion on any particular facts directly related to Seckinger's product other than teachings in the art in general, and his own considerable experience in the field, and does not take the SDS-PAGE into consideration. Affidavits or declarations are provided as evidence and must set forth facts, not merely conclusions.

I respectfully disagree with the statement of the examiner that I did not base my opinion on any particular facts directly related to Seckinger's product, other than teachings in the art in general. While I did provide a general background of the status of the field, and the evidence I cited in my declaration was provided against this background, my arguments were primarily derived from the papers by Seckinger et al., themselves, in combination and comparison with the competing papers by Engelmann/Wallach and Peetre/Olsson, all of which focus on and relate to the same TNF inhibitory protein. Thus, my conclusions were based on the facts as set forth in these papers. The arguments based on these papers are outlined in the sections from halfway through page 4 until halfway through page 5, and halfway through the first full paragraph on page 8 until the end of the last full paragraph on page 16, and thus comprise most of my first declaration. Respectfully, my opinions were all supported by the factual results and the statements in these papers, and not only by my personal experience.

That being said, it is indeed true that I did not discuss the possibility that a band could be sliced out of Seckinger's SDS-PAGE and a homogeneous protein eluted from a single band for sequencing, which the examiner states is well known in the art. Accordingly, I would like to address this possibility in the present declaration and explain why one of ordinary skill in the art would not believe that this would have been possible using the state of the art as of September 12, 1988, which I understand to be the U.S. filing date of the original application of which the above identified application claims benefit. My arguments below should make apparent why I did not discuss that possibility as a viable approach.

The following facts are relevant to an analysis of whether or not the bands of Seckinger's SDS-PAGE could be sliced out, and a homogeneous protein eluted and then sequenced.

Seckinger's Teaching About SDS-PAGE

The examiner refers to the teaching of Seckinger et al. (1988) relating to the presence of bands on SDS-PAGE. The only reference in Seckinger et al. (1988) to bands on SDS-PAGE appears in the first sentence of the Discussion, that reads:

> We have found that when tested in a cytotoxicity assay, urine from febrile patients contained a TNF-α inhibitory activity whose nature remains to be determined by purification to homogeneity,

> many bands being still identified in SDS-PAGE of the Sephacryl S-200 inhibitory fractions.

No mention is made of the conditions of SDS-PAGE that were used. The Materials and Methods section of Seckinger et al. (1988) is totally silent about SDS-PAGE. No gel is illustrated and no information is provided about the number or characteristics of the bands.

AS SDS-PAGE IS DENATURING, ONE CANNOT IDENTIFY WHICH BAND IS THE TNF INHIBITOR

Submitted herewith as Exhibit C (The exhibit letters are being continued from those previously submitted in my prior declaration) is Gallagher, "One-Dimensional SDS Gel Electrophoresis of Proteins", *Current Protocols in Molecular Biology*, 10.2A.1-10.2A.34 (1999) (hereinafter Gallagher A) and submitted herewith as Exhibit D is Gallagher, "One-Dimensional Electrophoresis Using Nondenaturing Conditions", *Current Protocols in Molecular Biology*, 10.2B.1-10.2B.11 (1999) (hereinafter Gallagher B). By definition, SDS-PAGE is denaturing. Note the second paragraph of Gallagher A, where it states that the standard Laemmli method is used for discontinuous gel electrophoresis "under denaturing conditions, that is, in the presence of sodium dodecyl sulfate (SDS)." The use of denaturing conditions involves (1) boiling the protein sample in sample loading buffer which contains up

to 2% SDS (see, for example Gallagher A (1999) (Exhibit C), page 10.2A.7, line 1, and 10.2A.28, "SDS sample buffer, 2x"; see also Garfin, "One-Dimensional Gel Electrophoresis," *Methods in Enzymology*, 182:425-441 (1990), submitted herewith as Exhibit E, page 433, "Sample Preparation," paragraph numbered 2, and page 430, "Stock sample buffer"), and (2) electrophoresing the proteins in a gel containing 0.1% SDS (see Gallagher A (1999) (Exhibit C), first paragraph). Note also the first paragraph in the right column of page 10.2A.29 of Gallagher A, where it states:

> In the separating gel, the glycine ions migrate past the proteins, and the proteins are separated according to either molecular size in a denaturing gel (containing SDS) or molecular shape, size, and charge in a nondenaturing gel.

The first paragraph of Gallagher B (Exhibit D) states:

> Because mobility depends on the size, shape, and intrinsic charge of the protein, nondenaturing electrophoresis provides a set of separation parameters distinctly different from mainly size-dependent denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; UNITS 10.2A)… .

Note also *Current Protocols in Molecular Biology*, vol. 2, Ausubel et al. eds., John Wiley and Sons, Inc., page 10.2.1 (1996) (Exhibit F), which relates to electrophoretic separation of proteins and states in the last sentence:

> If the protein is separated under denaturing conditions, any biological activity will

> likely be lost and, therefore, gel electrophoresis should definitely be the last purification step for a protein whose identity is based on a functional assay.

Current Protocols in Molecular Biology is considered to be a standard reference manual in the field of molecular biology and is considered by those of ordinary skill in the art to be factually accurate. The last above-quoted statement is in full concordance with my experience. In fact, the sample preparation prior to SDS-PAGE, i.e., boiling the protein at high SDS concentration, is already sufficient to fully denature the proteins, thereby destroying their biological activity. It is fully expected that any biological activity of a protein prepared for SDS-PAGE and separated under denaturing conditions will be lost, as is confirmed by the above quote from *Current Protocols in Molecular Biology* (Exhibit F).

In Seckinger et al. (1988), the identity of the protein is based on a functional assay. It is a previously unknown protein and its presence in a fraction is determined only by the functional assay of inhibiting TNF-α. The functional assay described in Seckinger et al. (1988) requires biological activity, which would be expected to be lost upon denaturation.

Even if, unexpectedly, the biological activity was not lost upon SDS-PAGE, there are other reasons why this functional assay on protein eluted from SDS-PAGE gel slices, would be expected to fail. Elution from an SDS-PAGE gel slice

releases SDS and toxic polyacrylamide gel components. The presence of these components in the biological sample for the functional assay will result in toxicity to the cells, thereby resulting in a high background of non-specific killing of the cells, independent of the presence of TNF or TNF Inhibitor in the assay.

Thus, as the presence of the protein can only be determined by a functional assay and a functional assay requires that the protein not be inactivated through denaturation, and as the presence of SDS and other gel components would be expected to affect the assay, it would be impossible to determine which of the many bands resulting from the SDS-PAGE referred to by Seckinger et al. (1988) might contain the protein of interest, even if it were present in a well defined band (which it would not be for the reasons to be discussed below).

Thus, even assuming that (1) each band contains only a single homogeneous protein (which cannot be assumed for the reasons that will be discussed below) and (2) there is a sufficient amount of protein in each band to allow sequencing with the techniques available to the art in 1988 (and there would not be sufficient TBP in any such band to allow sequencing for the reasons discussed below) and (3) there is sufficient TNF Inhibitor to permit staining so that it would be seen as a band (and it would not be expected that sufficient TBP would be present to allow one to see a distinct band containing it), one still would not have been able to

obtain a TNF-inhibitory protein of sufficient purity to allow determination of the N-terminal amino acid sequence thereof because one would have no idea which of the bands had TNF-inhibitory activity (if any).

THE IMPURITY OF THE PROTEIN CHARGED TO THE SDS-PAGE GEL MAKES IT VIRTUALLY IMPOSSIBLE TO OBTAIN A BAND ON SDS-PAGE CONTAINING HOMOGENEOUS PROTEIN CAPABLE OF BEING SEQUENCED

The amount of TBP Present in the Sample Prior to Sephacryl S-200 Fractionation

According to Seckinger et al. (1988), noting particularly the Materials and Methods section and the sentence bridging pages 1511 and 1512, 20 mg of ammonium sulfate-precipitated protein from urine of febrile patients was applied in 0.8 ml onto the Sephacryl S-200 column. Table 1 of Seckinger et al. (1990) shows that an 81,000-fold purification was required to obtain pure TNF Inhibitor from the same starting material. Thus, one can calculate that the sample applied to the S-200 Sephacryl column contained 0.25 µg TNF-inhibitor (20 mg/81,000). Assuming a molecular weight for the protein of interest of about 30 kDa (see page 25, lines 7-9, of the specification of the 10/036,434 application under examination, which states that the purified TNF-inhibitory protein moved as a single band with an apparent molecular weight of 26-28 kDa), this calculates to the presence of about 8 picomoles (pmol) of the protein of interest in the 20 mg sample of ammonium sulfate-precipitated protein that is loaded on the S-200 column.

The Amount Of Contaminating Protein in the S-200 Fractions

As stated on page 16 of my first declaration, Fig. 1 of Seckinger et al. (1988) shows a broad peak of biological activity that coincides with a substantial fraction of the protein. The peak of biological activity is comprised between fractions 15 and 21. To determine the amount of total protein that is present in these fractions, one must take the area beneath the curve of the total protein (the straight-line OD_{280} curve) that falls between fractions 15 and 21 and compare it to the area beneath the total curve (all fractions). Submitted herewith as Exhibit G is a copy of Figure 1 of Seckinger et al (1988) with the area beneath the total protein curve between fractions 15 and 21 shown with cross-hatching. One can see that the area beneath the curve between fractions 15 and 21 represents about 20 to 30% of the total curve. To be conservative, we have used the 20% figure. Thus, at least 20% of the total protein is eluted from this column with the peak of biological activity. Accordingly, the S-200 chromatography profile reflects a lack of efficient fractionation resulting in admittedly impure active fractions. Based on the fact that the activity peak corresponds to at least 20% of the protein, the fractionation results in no more than a 5-fold enrichment over the starting material. As the starting material contained 20 mg of proteins, the total protein in the fraction of interest was at best 4 mg.

The Amount of TNF Inhibitor Contained in Fractions 15-21 Representing the Activity Peak

In a manner similar to that discussed above for estimating the amount of total protein in fractions 15-21, one can also estimate the amount of total TNF Inhibitor within fractions 15-21, which represent the activity peak. Submitted herewith as Exhibit H is a copy of Figure 1 of Seckinger et al (1988) showing cross-hatching beneath the activity curve between fractions 15 and 21. One can see that no more than about 60% of the total biological activity eluted from the S-200 column is present in this activity peak. Thus, even if we were to assume a full recovery of all of the TNF Inhibitor biological activity following S-200 chromatography (which would be unrealistic as there is always loss), then the amount of TNF Inhibitor in the selected active fractions would still be no more than about 60% of the total. As about 8 pmol (about 0.25 µg) are loaded in the S-200 column, no more than about 4.8 pmol (about 0.15 µg) are present in the active fractions.

The Minimal Amount of Protein Required for N-Terminal Sequence Analysis in 1988

Tempst et al., "Internal Sequence Analysis of Proteins Separated on Polyacrylamide Gels at the Submicrogram level: Improved Methods, Applications and Gene Cloning Strategies", *Electrophoresis*, 11:537-553 (1990) (submitted herewith as Exhibit I) states in the first column of page 537, that, although some disagreement exists on the exact amount, approximately 5 pmol of purified protein is currently required

for analysis. It should be noted that the authors refer to the amount of protein immediately prior to the sequencing and not to the amount of protein of interest in the sample prior to fractionation or purification, which will result in substantial losses with each purification or recovery step. Aebersold et al., "Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after *in situ* protease digestion on nitrocellulose," *PNAS USA,* 84:6970-6974 (1987) (Exhibit J) reports in the second paragraph of the first column of page 6970 that, currently, 20 pmol amounts of protein can give limited N-terminal sequence information using optimized isolation methods. Aebersold et al., "Electroblotting onto Activated Glass: High Efficiency Preparation of Proteins from Analytical Sodium Dodecyl Sulfate-Polyacrylamide Gels for Direct Sequence Analysis," *J. Biol. Chem.*, 261:4229-4238 (1986) (Exhibit K), reports that amounts of protein in the 5-150 pmol range can be sequenced. Matsudaira, "Sequence from Picomole Quantities of Proteins Electroblotted onto Polyvinylidene Difluoride Membranes," *J. Biol. Chem.*, 262:10035-10038 (1987) (Exhibit L), reports that 7-250 pmol of protein can be sequenced. Furthermore, Moos, Jr., "Isolation of Proteins for Microsequence Analysis," *Current Protocols in Molecular Biology*, pp. 10.19.1-10.19.12 (1993), submitted herewith as Exhibit M, states at 10.19.11, first column, last full paragraph:

> In some cases, usable sequence may be obtained from as little as 10 pmol. In general, if a band is easy to see with Coomassie blue staining, there is enough to sequence, but if it is so faint that excision is difficult, it is doubtful that there is enough protein.

It should be emphasized that these numbers are based on model studies in which highly purified and well characterized proteins were used under optimized conditions, and not on an unknown protein being characterized for the first time.

Technical Limitations Related to SDS-PAGE

Two additional technical limitations should be understood and taken into consideration when analyzing the ability to sequence a protein subjected to SDS-PAGE. One is how much total protein can be loaded onto an SDS-PAGE gel column. The other is the amount of protein that must be present in order to be detectable by staining.

It is known that, compared to other protein purification procedures, SDS-PAGE represents a "low capacity step" (see Linn "Strategies and Considerations for Protein Purification", In *Guide to Protein Purification*, vol. 182, Deutscher, ed., Academic Press Inc., page 9 (1990) (Exhibit N). Gallagher A (1999) (Exhibit C), states, at page 10.2A.32, first column, second full paragraph, that, typically, 30 to 50 µg of a complex protein mixture in a total volume of <20 µl is loaded on a 0.75-mm-thick slab gel (16 cm, 10 wells).

See also, Tempst (1990) (Exhibit I), at page 540, right column, first paragraph, line 9, which states that no more than 50 µg total protein should be loaded in one well (1 cm) of a 1 mm thick mini gel. Loading larger amounts of protein results in loss of separation and quality due to problems such as band distortion and vertical streaking (see Gallagher A (1999) (Exhibit C), p. 10.2A.32, left column, last paragraph).

With respect to the amount of protein necessary to be detectable by staining, Gallagher A (1999) (Exhibit C) states that the protein of interest should be present in 0.2- to 1 µg amounts in a complex mixture of proteins if the gel will be stained by Coomassie Blue. See also Garfin (1990), Exhibit E, at page 433, where it states:

> Detection in gels requires on the order of 1 µg of protein per band for easy visibility when staining with Coomassie Blue R-250 or 0.1 µg of protein per band with silver staining … .

Accordingly, no more than about 50 µg of protein can be loaded onto an SDS-PAGE gel for proper resolution of the protein to be sequenced. Furthermore, at least 0.1 µg of protein is needed in order to detect a band of interest using even the most sensitive staining.

Insufficient TNF Inhibitor is Present in the Sephacryl S-200 Inhibitory Fraction to Yield a Band Capable of Being Sequenced

As discussed above, the material loaded on the S-200 column disclosed by Seckinger et al. (1988) contained at best 0.25 µg of TNF inhibitor and 20,000 µg of contaminating

protein. Following fractionation, the peak fractions contained, at best, 60% of the total TNF Inhibitor, i.e., 0.15 µg, and, at best, 20% of the total protein, i.e., 4,000 µg. A 50 µg aliquot of this fraction would contain only 1.25% (50/4000) of the total protein of interest, which is 1.8 ng of the protein of interest or 0.06 pmol of protein. This is substantially (>50-fold) less than the 100 ng that is the minimum amount of protein for even the most sensitive staining. It is also 80-fold less than the 5 pmol minimum amount required for sequencing under the best of conditions. In other words, loading a 50 µg sample of protein as prepared by Seckinger et al. (1988) would not allow visualization of the protein, let alone sequencing of the protein. This calculation does not even take into account that the preparation and recovery of proteins for sequence analysis by SDS-PAGE are not quantitative and will likely result in further loss of material.

There is No Assurance that Any Band Containing TNF Inhibitor will be Homogeneous

In a standard SDS-PAGE gel, if two proteins have similar molecular sizes, they more than likely will not be resolved (see Gallagher A (1999) (Exhibit C), p. 10.2A.30, first column, first full paragraph). Because of the inefficient fractionation in the S-200 column (at least 20% of total protein remains), and the very nature of S-200 gel filtration chromatography, which separates based on size, the

fractions containing the functional TBP will biochemically present themselves as complex mixtures of proteins with many proteins with similar sizes. Furthermore, abundant proteins will present migration patterns in gel that result in thick bands that will obscure proteins with much lower abundance. It is therefore to be expected that the mixture of proteins following S-200 fractionation will comprise proteins that are distinct from the TNF Inhibitor yet are sufficiently close in size to the TNF Inhibitor as to appear in the same band with it or in a band that is contiguous with it (assuming that there is enough TNF Inhibitor to be stained, contrary to the calculations above). The presence of more than one protein in a sample to be sequenced renders the obtained sequence not assignable to a single protein.

CONCLUSION

Accordingly, given the purity of the TNF Inhibitor in the Sephacryl S-200 fractions (estimated to be approximately 4.8 pmol in a total protein mixture of 4 mg) and the loading limitations of SDS-PAGE on a single lane (approximately 50 µg), it was not possible to load more than 1.8 ng (0.06 pmol) of TNF Inhibitor on a single lane. Thus, such a small amount of protein, even if it were to run as a compact homogeneous band (and there is no assurance of same),

could not be detected as a band by staining, since about 100 ng of protein is required for even the most sensitive stains according to Garfin (1990) (Exhibit E). Even if the band were detectable, the amount of protein was far below the limit (5 pmol) necessary for sequencing with the equipment in use in 1988. Indeed, it is 80-fold below the most sensitive lower limit for the amount of protein required for sequencing. Furthermore, it would have been impossible to determine which band contained the TNF inhibitor, since separation of the denatured proteins using SDS-PAGE would not allow a functional analysis of the proteins in the band.

For all of these reasons, it is my opinion, based on the facts presented herein, that one of ordinary skill in the art reading Seckinger et al. (1988) would not have been able to obtain the protein inhibitor of TNF-α in sufficient purity to allow determination of the N-terminal amino acid sequence regardless of the discussion therein relating to SDS-PAGE.

The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such

willful false statements may jeopardize the validity of the application or any patent issued thereon.

June 18, 2007
Date

/Rik Derynck/
Rik Derynck, Ph.D.

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